

Field and Laboratory Studies of the Etiology of Liver Neoplasms in Marine Fish from Puget Sound

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A series of field studies was conducted between 1979 and 1985 in Puget Sound, Washington State, to investigate etiological relationships between prevalences of hepatic neoplasms in bottom-dwelling marine fish species, with emphasis on English sole (*Parophrys vetulus*), and concentrations of toxic chemicals in sediments and affected fish. Statistically significant ($p \leq 0.05$) correlations have been found between the prevalences of hepatic neoplasms in English sole and the following parameters: (1) sediment concentrations of aromatic hydrocarbons, and (2) concentrations of the metabolites of aromatic compounds in the bile of affected sole. A significant difference ($p < 0.001$) was also found between the relative concentrations of aromatic free radicals in the liver microsomes of English sole with liver lesions compared to sole without liver lesions. Laboratory studies designed to evaluate the etiology of the liver neoplasms in English sole have also yielded evidence that is consistent with the view that high molecular weight aromatic hydrocarbons, e.g., benzo[a]pyrene (BaP), are hepatocarcinogens in English sole. The current status of a series of long-term (up to 18 months) exposures of English sole and rainbow trout (*Salmo gairdneri*) to selected fractions of Puget Sound sediment extracts, enriched with aromatic hydrocarbons and nitrogen-containing aromatic compounds, and to individual carcinogens (e.g., BaP) is discussed.

Introduction

A 1975 study of Puget Sound, Washington State, revealed that bottom-dwelling fish from Seattle's Duwamish River had a variety of diseases of the liver, including neoplasia (1). Subsequent multidisciplinary studies, conducted between February 1979 and December 1984, showed that high levels of toxic chemicals in sediments and serious diseases in bottom-dwelling fish in Puget Sound were not confined to the Duwamish River (2-4). In fact, neoplasms and other liver diseases were shown to occur in high prevalences in bottom-dwelling fish living in waters adjacent to various urban (industrialized) areas. These studies also indicated that the hepatic neoplasms were closely associated with the presence of elevated levels of such toxic chemicals in the sediments as aromatic hydrocarbons (AHs) and metals. This association between certain chemicals and liver neoplasia has been further studied in the field and laboratory.

The objectives of this paper are to review field investigations concerned with the nature and distribution of liver neoplasms in bottom-dwelling fish and sediment-associated chemicals in Puget Sound and field and laboratory studies that address the etiology of the neoplasms.

Field Investigations

A main focus of the Puget Sound work involved multidisciplinary field studies of relationships between sediment-associated chemicals and diseases, such as neoplasms, in bottom-dwelling fish. These investigations were conducted in urban (industrialized) and nonurban (essentially non-industrialized) areas of Puget Sound. The major sampling areas included Elliott Bay (Seattle), Commencement Bay (Tacoma), Sinclair Inlet (Bremer-ton), Possession Sound (near Mukilteo), Eagle Harbor (near Winslow), and Port Gardner (Everett); the reference areas within Puget Sound were Case Inlet and Port Madison (Fig. 1).

In most cases, sediment and English sole (*Parophrys vetulus*), rock sole (*Lepidopsetta bilineata*), and Pacific staghorn sculpin (*Leptocottus armatus*) were obtained from the various urban and nonurban areas. Sediment

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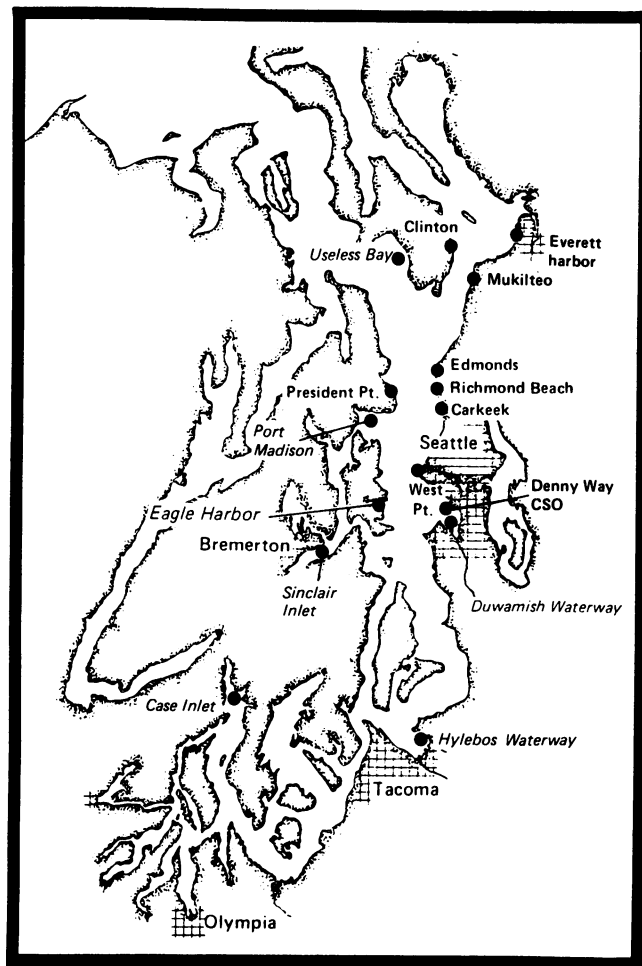


FIGURE 1. Map of Puget Sound showing selected sampling sites.

and stomach contents were analyzed for AHs; polychlorinated biphenyls (PCBs), chlorinated pesticides, and other chlorinated organic compounds; and metals (Table 1). Fish liver and skeletal muscle were analyzed for chlorinated compounds only. Analyses for organic compounds employed column chromatography in conjunction with capillary gas chromatography/mass spectrometry (GC/MS) (5). Analyses for metals were by either atomic absorption spectroscopy or inductively coupled argon plasma emission spectroscopy (6). Concentrations of aromatic compounds (metabolites) in the bile of English sole were measured by using HPLC/fluorescence (7,8). Histological techniques used are detailed elsewhere (9,10).

Chemicals in Sediments and Fish

A large number and a wide variety of chemicals were found in the Puget Sound sediments. For example, in Commencement Bay more than 900 individual organic compounds were detected, including over 500 AHs, hundreds of chlorinated hydrocarbons, as well as various compounds containing nitrogen, oxygen, sulfur,

Table 1. Chemicals analyzed in sediment and biota from Puget Sound.

| Aromatic hydrocarbons | Chlorinated organic compounds | Metals and other elements |
|----------------------------|-------------------------------|---------------------------|
| Acenaphthene | Aldrin | Aluminum |
| Anthracene | α -Chlordane | Antimony |
| Benz[a]anthracene | <i>m,p'</i> -DDD | Arsenic |
| Benzo[a]pyrene | <i>o,p'</i> -DDD | Barium |
| Benzo[e]pyrene | <i>p,p'</i> -DDD | Beryllium |
| Benzo[fluoranthene] | <i>o,p'</i> -DDE | Bismuth |
| Biphenyl | <i>p,p'</i> -DDE | Boron |
| Chrysene | <i>o,p'</i> -DDT | Cadmium |
| Dibenzanthracene | <i>p,p'</i> -DDT | Calcium |
| 2,6-Dimethylnaphthalene | Dichlorobiphenyls | Chromium |
| 3,6-Dimethylphenanthrene | Dichlorobutadienes | Cobalt |
| Fluoranthene | Heptachlor | Copper |
| Fluorene | Heptachlorobiphenyls | Gallium |
| Indan | Hexachlorobenzene | Germanium |
| Isopropylbenzene | Hexachlorobiphenyls | Iron |
| 1-Methylnaphthalene | Hexachlorobutadiene | Lead |
| 2-Methylnaphthalene | Lindane | Lithium |
| 1-Methylphenanthrene | Nonachlorobiphenyls | Magnesium |
| Naphthalene | Octachlorobiphenyls | Manganese |
| <i>n</i> -Propylbenzene | Pentachlorobiphenyls | Mercury |
| Perylene | Pentachlorobutadienes | Molybdenum |
| Phenanthrene | Tetrachlorobiphenyls | Nickel |
| Pyrene | Tetrachlorobutadienes | Phosphorus |
| 1,2,3,4-Tetramethylbenzene | <i>trans</i> -Nonachlor | Potassium |
| 2,3,5-Trimethylnaphthalene | Trichlorobiphenyls | Scandium |
| | Trichlorobutadienes | Selenium |
| | | Silicon |
| | | Silver |
| | | Sodium |
| | | Strontium |
| | | Tin |
| | | Titanium |
| | | Tungsten |
| | | Vanadium |
| | | Yttrium |
| | | Zinc |
| | | Zirconium |

and bromine (6). There were indications that many other chemicals were present (especially in the Everett Harbor); however, their numbers and identities were not elucidated because of the limitations of the analytical techniques.

Mean concentrations of AHs in sediment (all sediment concentrations are expressed on a dry weight basis) from urbanized and/or industrialized sites—Commencement Bay, Elliott Bay, Sinclair Inlet, Eagle Harbor, Mukilteo, and Everett Harbor—were at least 150 times higher than the mean concentration in sediments from the nonurban embayments—Case Inlet and Port Madison (Fig. 2). Within these urban areas, the values varied considerably with respect to individual stations (e.g., 150 to 63,000 ppb in Elliott Bay). In Elliott Bay, the highest concentrations of summed AHs were found in sediments from a station adjacent to a combined sewer overflow (CSO) on Seattle's waterfront and from a station slightly north of a sewage treatment facility (north of Seattle). Sediments from the former station also had the highest mean concentrations of the carcinogenic compound benzo[a]pyrene (BaP, 2400 ± 280 ng/

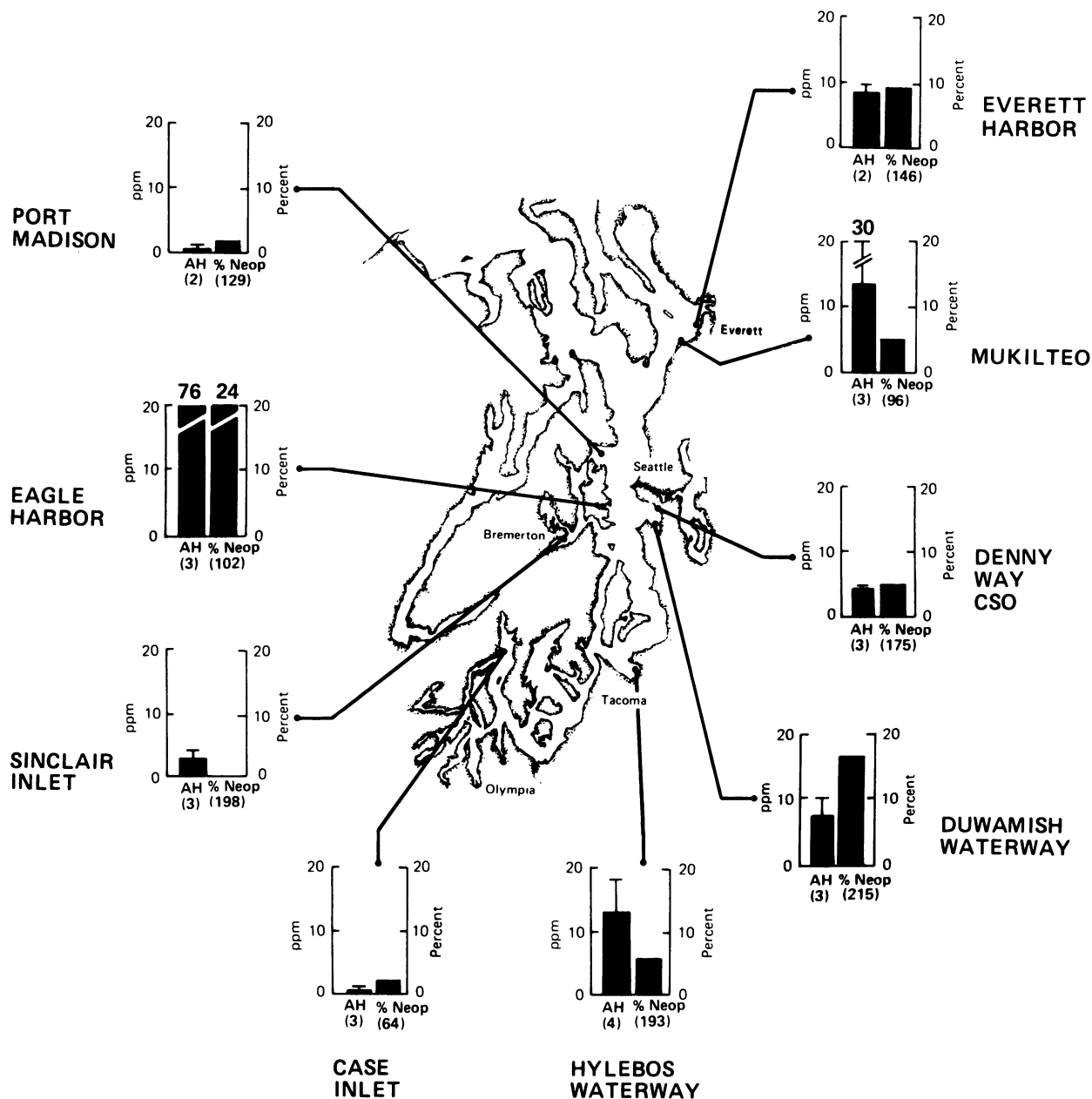


FIGURE 2. Mean concentrations (in ppm dry weight \pm SD) of aromatic hydrocarbons (AH) [(n) under bars indicates number of sediment samples analyzed per site] in sediments, and prevalences (%) of hepatic neoplasms in English sole [(n) under bars indicates number of fish examined] from selected sites in Puget Sound. Samples were collected and analyzed between 1979 and 1985. Data taken in part from references (2-4,6).

g) found in Elliott Bay. High concentrations of summed AHs (120,000 ng/g) were also detected in sediments from Eagle Harbor, as were over 200 nitrogen-containing aromatic compounds (4,11). Representative nitrogen-containing aromatic compounds (NCAC), carbazole, 1-cyanonaphthalene, acridine, and benzo[a]carbazole were found at concentrations of 18,000, 17,000, 6,800, and 17,000 ng/g, respectively, in one highly contaminated sediment sample (11). This large number of nitrogen-containing aromatic compounds found in the sediment is far greater than has

been previously identified in any marine sediment. The composition of the aromatic compounds was characteristic of cresote. The mean concentrations of BaP and carbazole from the nonurban areas were each less than 25 ppb.

In addition to aromatic compounds and polychlorinated biphenyls (PCBs), various chlorinated butadienes (CBDs) were found in virtually every sediment sample (Fig. 3). Concentrations of PCBs were usually much higher in sediments from most of the sites in Elliott and Commencement Bays compared to those in sediments

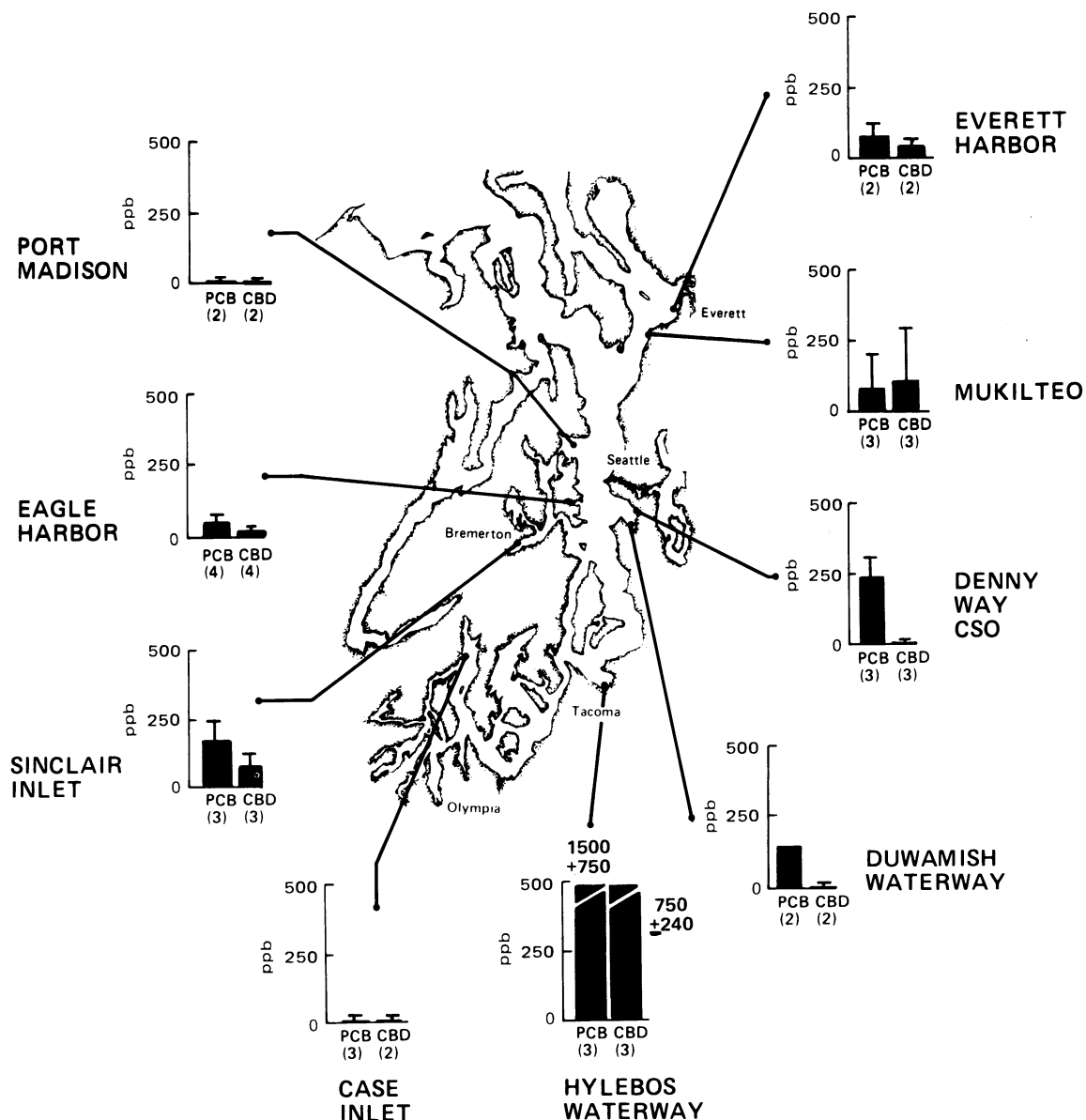


FIGURE 3. Mean concentrations (in ppb dry weight \pm SD) of polychlorinated biphenyls (PCB) and chlorinated butadienes (CBD) [(n) under bars indicates number of sediment samples analyzed per site] in sediments from selected sites. Samples were collected and analyzed between 1979 and 1985. Data taken in part from references (2-4,6).

from sites in nonurban areas, and the concentrations of CBDs were substantially higher in Commencement Bay. The mean concentrations of metals with known carcinogenic properties, such as cadmium and arsenic (12), were also of interest. Arsenic concentrations were consistently higher in the major urban areas (Sinclair Inlet and Commencement and Elliott Bays) than in the nonurban areas, whereas the mean concentrations of cadmium were generally similar in all areas.

Food organisms in the stomachs of the English sole from selected areas, such as Eagle Harbor and near Mukilteo, contained substantially higher concentrations of aromatic hydrocarbons than comparable organisms from reference sites. For example, the sums of the con-

centrations of AHs in two composites of food organisms (from five fish each) from Eagle Harbor were 50,000 and 84,000 ng/g, respectively. The concentrations of individual AHs in a composite of benthic food organisms (from six fish) from President Point did not exceed 100 ng/g.

Metabolically resistant organic chemicals, such as PCBs, hexachlorobutadiene (HCB), and hexachlorobenzene (HCB), were found in the muscle and liver tissues of English sole at concentrations higher than those found in sediments from their respective collection sites. For example, the mean concentration of PCBs in sediments from the Duwamish Waterway was 510 ± 30 ng/g (Fig. 2), whereas mean concentrations of PCBs

in liver and muscle of English sole from this waterway were $47,000 \pm 25,000$ ng/g ($n = 5$) and 4800 ± 3400 ng/g ($n = 5$), respectively (2). In contrast, concentrations of the more metabolically labile organic compounds (e.g., AHs) (13–16) were generally below the limits of detection in the muscle and liver of English sole captured in areas with highly contaminated sediment. Analyses of samples from the Hylebos Waterway in Commencement Bay yielded a representative example: mean concentrations of summed AHs in the sediments were $13,600 \pm 5300$ ng/g (Fig. 2); however, no AHs were detected in the liver or muscle of English sole ($n = 5$) from this waterway (2).

Because AHs are rapidly metabolized by fish, new HPLC-fluorescence techniques were developed in our laboratories which allowed measurement of relative concentrations of metabolites of aromatic compounds in fish bile (7,8). In a study of English sole captured from 11 sites in Puget Sound (8), high concentrations of metabolites which fluoresce at the BaP wavelength pair (380/430 nm) were found in bile of fish from Eagle Harbor (2100 ± 1500 ng/g) and the Duwamish Waterway (1400 ± 2200 ng/g), compared to reference sites where concentrations were at least 20 times less (Fig. 4).

The bile and liver of English sole were also analyzed for free radicals by electron spin resonance (ESR) spectrometry (Fig. 5). Numerous studies with mammalian species have demonstrated that free radicals may play a crucial role in chemical carcinogenesis (17). The concentrations of xenobiotic free radicals in liver correlated significantly ($p < 0.001$) with concentrations of metabolites of aromatic compounds in bile. The liver concentrations of total xenobiotic free radicals reflected levels of contaminants in the sediments from where the fish were captured. For example, livers of fish from a heavily polluted area near the Denny Way CSO (Fig. 1) contained the highest accumulations of free radicals, expressed as carbazole-9-oxyl, (~ 18 ng/g, wet weight), whereas fish from a nonurban area, Case Inlet (Fig. 1), contained the lowest liver concentration (~ 1 ng/g, wet weight) (18,19). No xenobiotic free radicals have thus far been detected in muscle tissue from English sole.

Diseases in Bottom-Dwelling Fish

The liver lesions in bottom-dwelling fish were classified by histopathological features. Neoplasms included carcinomas (hepatocellular, cholangiocellular and mixed hepatocellular/cholangiocellular), adenomas (liver cell adenoma and cholangioma), and hepatic mesenchymal neoplasms (hemangioma, fibroma) (Figs. 6–12). The morphology and occurrence of the nonneoplastic liver lesions are described elsewhere (6,20).

The highest prevalences of hepatic neoplasms were found in English sole from the lower Duwamish Waterway in Elliott Bay and Eagle Harbor (Fig. 2). By comparison, the prevalences of hepatic neoplasms in English sole from Everett Harbor, Mukilteo, and the Hylebos Waterway in Commencement Bay ranged from 5 to 9% (Fig. 2). With respect to hepatic neoplasms in rock sole,

the areas having the highest lesion prevalences were in the Everett Harbor (4.7%, $n = 43$) and Commencement Bay's Hylebos Waterway (2.5%, $n = 159$). Hepatic neoplasms were found in rock sole from all of the urban embayments at prevalences from 0.7 to 4.7%. Pacific staghorn sculpin with hepatic neoplasms were found only in Commencement Bay (1.7%, $n = 116$). In the 1979/1980 studies, hepatic neoplasms were not found in fish ($n = 260$) from any nonurban areas studied (e.g., Case Inlet and Port Madison). However, in subsequent studies conducted in 1984 and 1985, liver neoplasms were detected in English sole from Case Inlet and Port Madison (Fig. 2).

Results of statistical analyses of relationships between prevalences of liver neoplasms in English sole and factors such as age, gender, season, and year of capture of affected fish demonstrated that older fish tended to have a higher risk of liver neoplasms, whereas the other factors did not have a significant influence (21). In addition, statistical analysis of the patterns of co-occurrence of liver lesions in English sole suggests that the progressive sequence of lesions leading to liver neoplasms is similar to that documented in laboratory rodents exposed to various hepatocarcinogens (22).

Associations between Chemicals and Neoplastic Lesions in English Sole

Because of the consistently demonstrated association of liver neoplasms in English sole with chemically contaminated environments (1,3,4), relationships between prevalences of liver neoplasms and concentrations of certain groups of chemicals in bottom sediments and fish tissues were investigated (2). In order to simplify the complex data set on sediment concentrations of 28 metals and 36 AHs and chlorinated hydrocarbons (CHs) (Table 1) from 40 sampling stations at which both fish and sediment were collected, factor analysis was performed. This mathematical method for sorting into groups chemicals whose concentrations in sediments correlate positively with each other yielded four major factors (groups) which accounted for 75% of the variance in the concentrations of chemicals among the stations. Group 1 was dominated by the AHs except for isopropylbenzene, 1,2,3,4-tetramethylbenzene, 1-methylphenanthrene, 3,6-dimethylphenanthrene, and dibenzanthracene. Group 2 was dominated by the metals, except Hg, Al, Ba, Bi, B, Ca, Ga, Mg, K, Na, Sr, and Mn. Group 3 was dominated by PCBs and Ti, Sc, Y, Li, Be, Ba, V, and Mn, and Group 4 by the CHs, except Lindane, heptachlor, aldrin, *p,p'*-DDD, and *p,p'*-DDT. Summed concentrations of each of these four groups of xenobiotics were used in subsequent statistical analyses of the data.

Using the Spearman rank correlation procedure, a significant ($p = 0.003$) positive correlation ($r_s = 0.48$) was found between the prevalence of hepatic neoplasms in English sole and sediment concentrations of AHs (first group). A weaker correlation ($r_s = 0.37$) with hepatic neoplasms in this species was obtained with met-

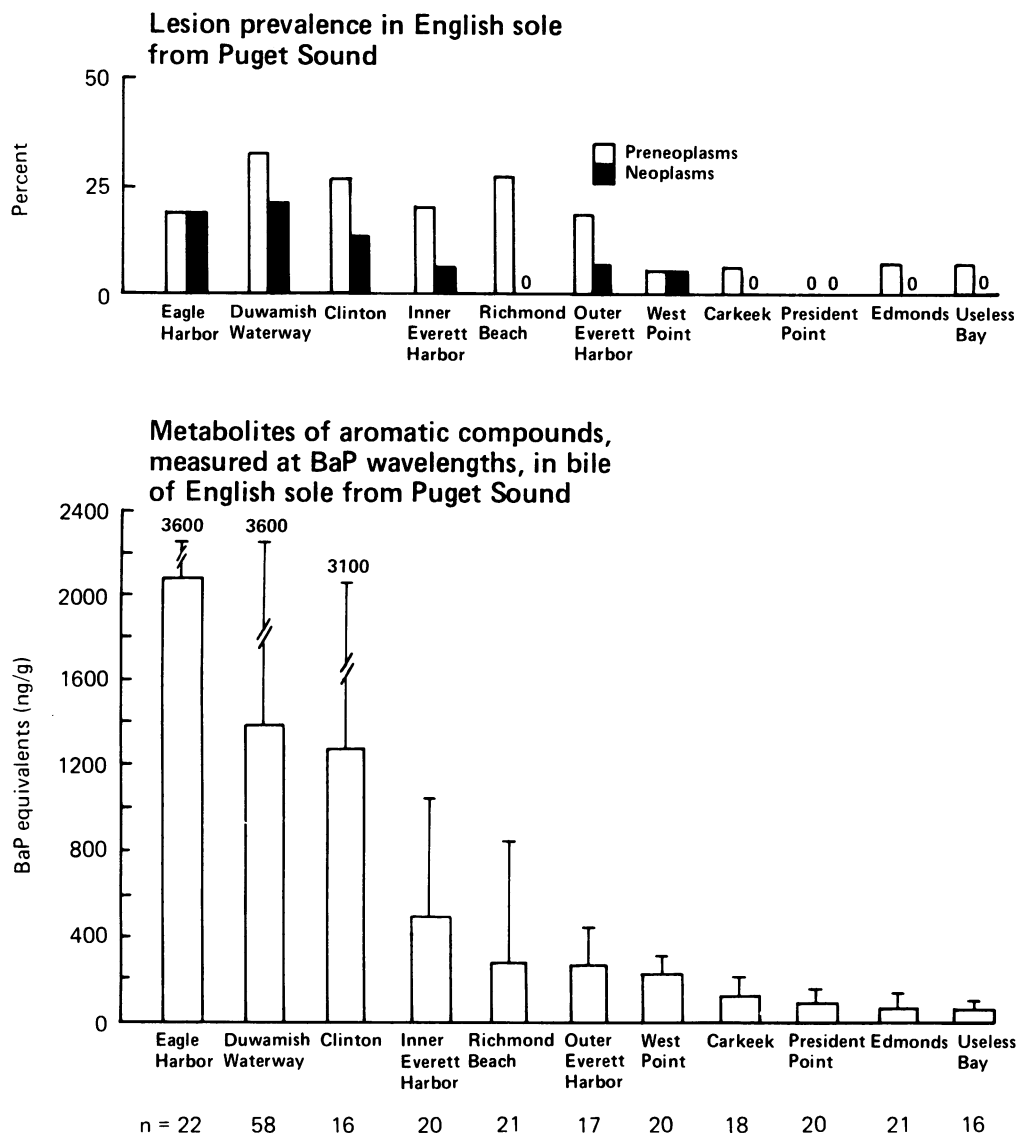


FIGURE 4. Comparisons between bile concentrations of aromatic compounds (measured at BaP wavelengths) and prevalences of hepatic neoplasms and "preneoplasms" in English sole from 11 sites in Puget Sound. Data from Krahn et al. (8).

als (second group) ($p = 0.019$). Importantly, no significant negative correlations were found. Moreover, in a recently completed summary analysis of the data from the original study of 40 stations combined with data from subsequent studies of 30 stations (i.e., including stations in Eagle Harbor and near Mukilteo), a significant ($p = 0.0001$) positive correlation between concentrations of sediment-associated AHs was found using the Spearman rank correlation.

Although, as indicated, positive correlations were obtained between the prevalence of hepatic neoplasms and certain individual groups of sediment-associated chemicals, many sampling stations had sediment with high concentrations of several groups of chemicals. For this reason, lesion prevalences were also compared in fish from sampling stations grouped by cluster analysis on the basis of similar sediment concentrations of several

groups of chemicals. The highest prevalences of hepatic neoplasms in English sole were found in two of the eight clusters—the stations in the cluster that represented the Duwamish Waterway and the cluster station that represented the Hylebos Waterway.

Bile metabolite concentrations measured (at the BaP wavelength pair) in English sole from 11 sites in Puget Sound (8) were also compared statistically (Spearman rank correlation) to the prevalences of certain types of hepatic lesions, including neoplasms in these fish. A significant ($p < 0.002$) positive correlation ($r_s = 0.85$) was found. These results provide supportive evidence for the putative relationship between the aromatic compounds found in the environment and serious liver diseases in bottom-dwelling fish, such as the hepatic neoplasms.

Using ESR spectroscopy, it was also found that mi-

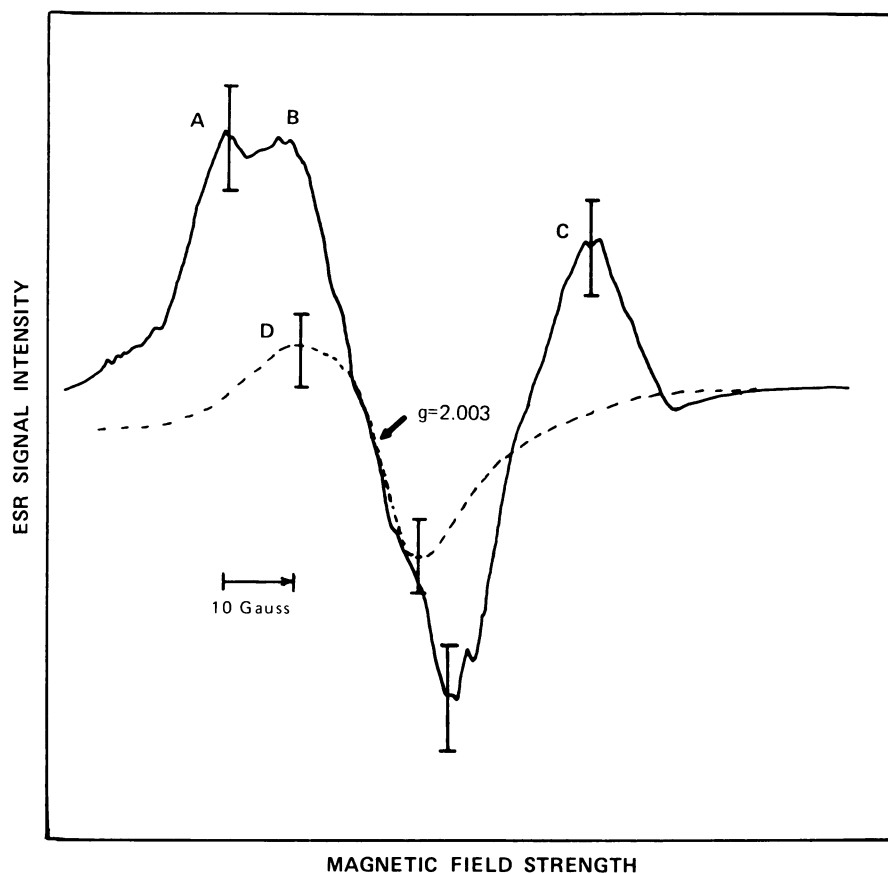


FIGURE 5. ESR spectra for lyophilized liver microsomes from English sole: (—) spectrum for free radicals in samples containing idiopathic liver lesions (Duwamish River fish); (---) the single resonance is the spectrum for free radicals in liver samples devoid of lesions (Meadow Point fish). The spectra were recorded at room temperature. Taken from Malins et al. (18).

microsomes from livers of English sole with lesions (including hepatic neoplasms) had concentrations of free radicals which were significantly higher ($p < 0.001$) compared to microsomes from livers without lesions (18). The free radicals identified in livers with lesions included *N*-oxyl derivatives of carbazoles, which appear to be derived metabolically from exposure of fish to carbazoles in the environment. Many of these nitrogen-containing compounds, which "overlap" with the polynuclear AHs in GC/MS analyses, are of considerable interest in broadening our understanding of the etiology of the hepatic neoplasms.

In considering relations between environmental contaminants and the hepatic neoplasms, the question of whether the bottom-dwelling fish are moving in and out of polluted areas is important. Although migration or movement patterns of the three fish species are largely unknown, English sole (23) and rock sole (24) are essentially territorial, forming localized discrete subpopulations. While adult sole do make limited seasonal migrations for spawning (in winter), they apparently return to their home territory. Less is known about the migratory behavior of Pacific staghorn sculpin, although limited studies do suggest that they are also territorial (25). Accordingly, it appears that these three species of

bottom-dwelling fish reside in polluted areas for relatively long, though presently undefined, periods of time.

Laboratory Studies

Statistically significant correlations between chemicals in sediment and liver neoplasms in the bottom-dwelling fish appear to be indicative of a general cause-and-effect relationship. However, such relationships cannot be interpreted as *de facto* evidence of specific cause and effect. One obvious and inherent limitation is that not all sediment-associated chemicals can presently be identified; thus unidentified compounds cannot be ruled out as principal etiological factors in the liver neoplasia. Also, the classes of chemicals which correlate with the liver neoplasia may act through synergistic/antagonistic interactions, thus making the elucidation of cause-and-effect relationships even more difficult. Controlled laboratory tests were clearly necessary to further understand the apparent relationships obtained thus far.

Because the above-mentioned field studies found positive correlations between the prevalences of hepatic neoplasms in English sole and concentrations of high molecular weight AHs in sediment and metabolites of



FIGURE 6. Gross appearance of intrahepatic neoplastic nodules in an English sole from the Duwamish Waterway, Seattle, WA. These lesions were hepatocellular carcinoma and cholangiocellular carcinoma.

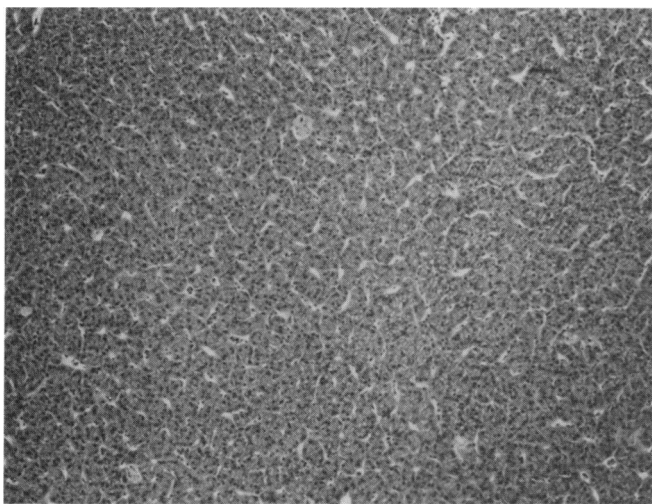


FIGURE 7. Normal English sole liver tissue, showing uniform staining characteristics and typical muralial architecture. Hematoxylin and eosin, 130 \times .

aromatic compounds (BaP equivalents) in bile, BaP has been the subject of extensive study in our laboratories as a "model" carcinogenic hydrocarbon. Detailed results of these studies are presented elsewhere in this volume. In one study, in which radiolabeled BaP was orally administered to English sole, a relatively high percentage of BaP 7,8-diol (the penultimate carcinogen of BaP) (26,27) was found in the liver. Moreover, a higher percentage of this metabolite and a greater degree of cov-

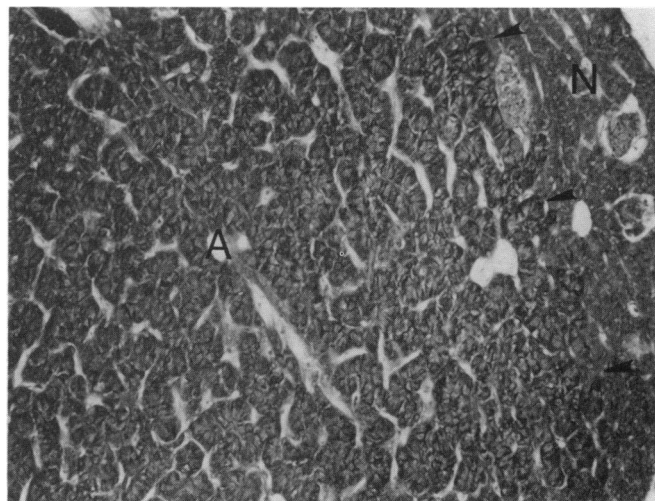


FIGURE 8. The highly basophilic, well-organized liver cords are part of a liver cell adenoma (A) in an English sole which compresses the adjacent normal hepatic parenchyma (N). Arrows delineate the margin of the neoplastic tissue. Hematoxylin and eosin, 130 \times .

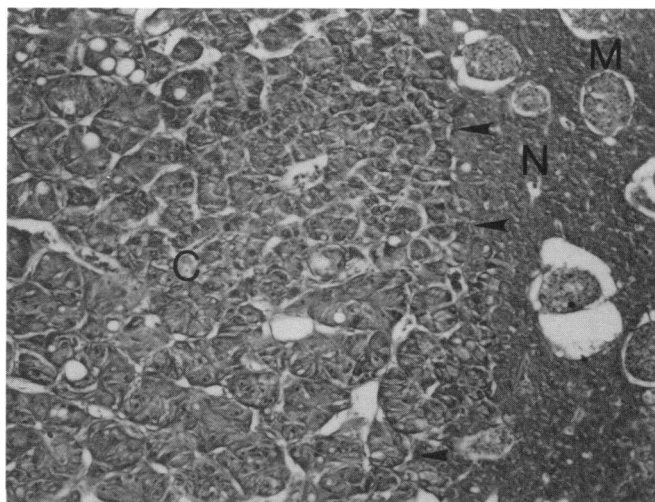


FIGURE 9. The basophilic, poorly organized liver cords are composed of enlarged, pleomorphic, anaplastic hepatocytes which have lost the typical polar orientation to the sinusoids. This hepatocellular carcinoma (C) is from an English sole from the Duwamish Waterway in Seattle, WA. Arrows delineate the margin of the carcinoma; normal parenchyma is identified (N), as are melanomacrophage centers (M). Hematoxylin and eosin, 130 \times .

alent binding to DNA occurred in English sole liver compared to mouse liver (28). The high degree of DNA binding observed with the liver of English sole exposed to a single dose of BaP persists for several weeks, suggesting that excision-repair of modified DNA may be slow in these fish.

Other laboratory studies in progress involve a series of long-term exposures of English sole and rainbow trout to selected fractions of Puget Sound sediment extracts and to selected individual compounds known to be carcinogenic in laboratory mammals. The sediment

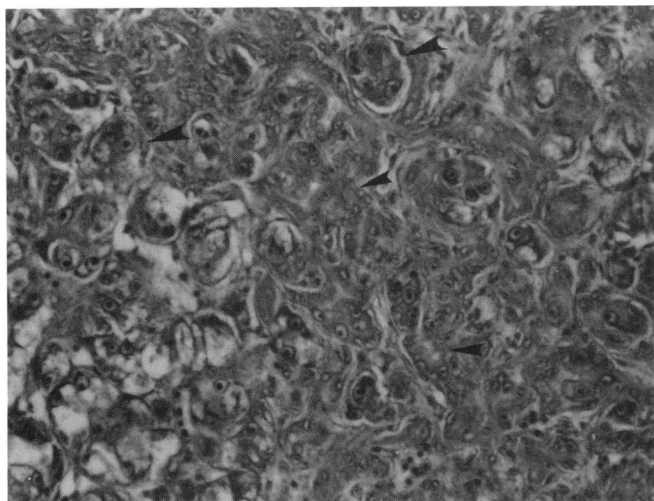


FIGURE 10. This mixed cholangio/hepatocellular carcinoma in an English sole clearly shows the separate components. The larger cells with prominent nuclei are neoplastic hepatocytes (large arrows), while the small cells with small nuclei which are often arranged in a tubular pattern form the cholangiocellular component (small arrows). Hematoxylin and eosin, 245 \times .

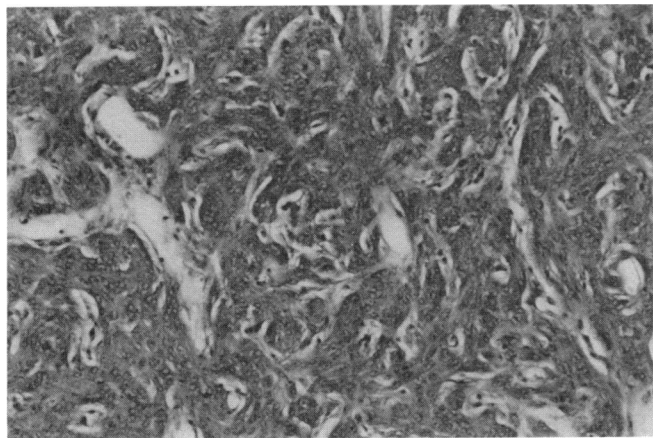


FIGURE 11. This poorly differentiated cholangiocellular carcinoma in an English sole metastasized to the heart, kidney, spleen and muscle wall of the small intestine. Hematoxylin and eosin, 195 \times .

extracts under investigation include those from Eagle Harbor and a reference site. As mentioned above, Eagle Harbor is a mixed residential and industrialized embayment containing extremely high concentrations of creosote-like hydrocarbons (4). In contrast, few detectable xenobiotics were found in sediment from the reference site. A critical first step in the conduct of these studies was the development of laboratory protocols to extract, fractionate and chemically characterize the organic contaminants of these sediments. This has been accomplished and fractions characterized by >95% high molecular weight AHs and >95% NCAC have been prepared for testing (Table 2) (11).

Carcinogenicity tests in progress using English sole include two long-term (up to 18 months) studies in which

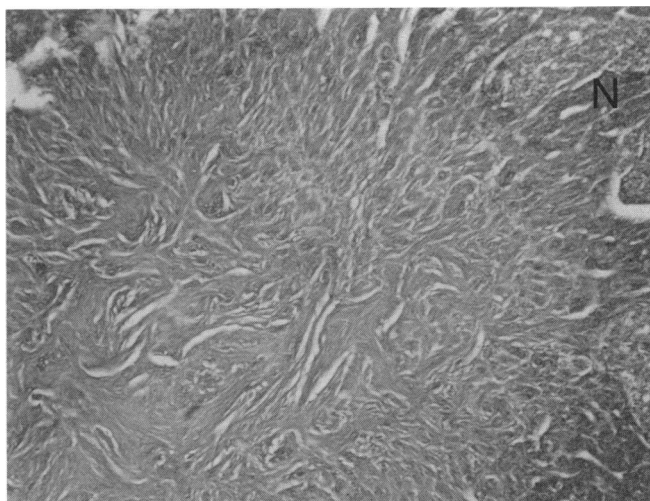


FIGURE 12. This heavily collagenized mesenchymal neoplasm has irregular margins and is invading the adjacent hepatic parenchyma. Due to the invasion and the disorganized architecture of the neoplasm, diagnosis was a fibrosarcoma. Normal hepatic parenchyma is indicated (N). Hematoxylin and eosin, 130 \times .

groups of juvenile fish are being exposed to a combined high molecular weight AHs and NCAC fraction of Eagle Harbor sediment extract (Table 2) or a similar combined fraction from the reference sediment extract via intramuscular injection. A similar experiment is also being conducted utilizing a dietary route of exposure. Future studies are planned to evaluate the carcinogenicity of the fractions of AHs and NCAC on an individual basis. In the experiment utilizing parenteral exposure, sole receive intramuscular injections of sediment extracts (doses of 30 or 75 mg/kg), BaP (dose of 12 mg/kg), or control solutions at 4-week intervals. In the dietary study, sole are fed a pelletized diet the same sediment extracts at concentrations that yield overall doses comparable to those administered in the parental exposure.

Carcinogenicity tests in progress utilizing rainbow trout include one being conducted in a parallel fashion to the English sole parenteral exposure study. Trout are exposed at 4-week intervals via intramuscular injection to the combined high molecular weight AH and NCAC fraction of either Eagle Harbor or reference sediment extracts (doses of 50 and 150 mg/kg) as well as to BaP (dose of 25 mg/kg). We are presently examining histological sections from trout sacrificed after 6 months of exposure.

The combined high molecular weight AHs and NCAC fractions of Eagle Harbor sediment extract and reference sediment extract were tested for mutagenicity using the Ames Salmonella/mutagenicity assay (29). The combined Eagle Harbor sediment extract fraction was found to be mutagenic in two Salmonella tester strains (TA 98 and TA 100) when metabolically activated; a twofold and dose-related increase in colony counts was observed when compared to solvent controls. In contrast, the reference sediment extract fractions exhibited no mutagenic activity.

Table 2. Concentrations of selected aromatic hydrocarbons and nitrogen-containing aromatic compounds in two fractions of extracts from Eagle Harbor sediment.

| Compound | Concentration, ng/ μ L ^{a,b} | |
|-----------------------------------|---|------------|
| | Fraction 1 | Fraction 2 |
| Naphthalene | 2.2 | — |
| 2-Methylnaphthalene | 1.8 | — |
| Biphenyl | 1.7 | — |
| 2,6-Dimethylnaphthalene | 8.0 | 0.9 |
| Acenaphthene | 82 | 4.5 |
| Fluorene | 110 | 5.1 |
| Phenanthrene | 220 | 10.2 |
| Anthracene | 68 | 7.2 |
| 1-Methylphenanthrene | 20 | 12 |
| Fluoranthene | 500 | 15 |
| Pyrene | 220 | 9.6 |
| Benz[a]anthracene | 85 | — |
| Chrysene | 120 | — |
| Benzo[e]pyrene | 28 | — |
| Benzo[a]pyrene | 24 | — |
| Perylene | 10 | — |
| Dibenzanthracene | 5.3 | — |
| 7-and/or 6-Methylquinoline | 2.0 | — |
| 2,7- and/or 2,6-Dimethylquinoline | — | 6.0 |
| 2,4-Dimethylquinoline | — | 7.8 |
| 1-Cyanonaphthalene | 34 | 29 |
| 2-Cyanonaphthalene | — | 6.0 |
| 7,8-Benzoquinoline | — | 4.2 |
| Acridine | — | 33 |
| Carbazole | 56 | 28 |
| 2-Methyl-5,6-benzoquinoline | — | 23 |
| 1-Methylcarbazole | 23 | 13 |
| 3-Methylcarbazole | 10 | 11 |
| 2- and/or 4-Methylcarbazole | 20 | 13 |
| 9-Cyanoanthracene | 3.8 | — |
| 9-Cyanophenanthrene | 2.7 | — |
| Benzo[a]carbazole | 4.2 | 32 |

^aSolutions of these concentrations and 1,2,3,5 and 10-fold greater concentration are used in laboratory exposure studies.

^bThe dash (—) indicates that the chemical was not detected. The detection limit for aromatic hydrocarbons is 1.0 ng/ μ L (GC/FID) and for nitrogen heterocycles is 1.2 ng/ μ L (GC-nitrogen specific detector). All values are the mean of two analyses.

An additional test of the carcinogenicity of selected compounds and fractions of Puget Sound sediment extracts is underway utilizing a recently developed rainbow trout embryo microinjection assay (30). The carcinogenicity of both the high molecular weight AH and NCAC fractions of Eagle Harbor and reference sediment extracts are being evaluated alone and in combination. Also being evaluated are BaP and carbazole. In this assay, trout embryos are exposed via a single injection of test solution into the yolk sac approximately 1 week before hatching. At regular intervals following hatching, the fish are being sampled and evaluated histologically for evidence of neoplastic and preneoplastic liver lesions.

Conclusions and Implications

Comparisons of prevalences of liver neoplasms with sediment-associated chemicals have been of considerable value for identifying presumptive factors in the eti-

ology of these lesions in English sole. The significant statistical correlation between sediment-associated AHs and prevalences of liver neoplasms in English sole established in our Puget Sound studies provided such presumptive etiological evidence. This finding has been further substantiated by the results of subsequent field studies, especially the strong statistical correlation which was found between bile concentrations of aromatic metabolites and liver neoplasms in English sole (8). Although the selected bile metabolites that were measured reflect relatively recent uptake of aromatic compounds, they provide evidence that these fish are being exposed to known carcinogens.

Other evidence supporting the etiological role of AHs came from the studies in Eagle Harbor and near Mukilteo (3,4). Sediments from these two areas had high concentrations of AHs, including known mutagens and carcinogens, as did the stomach contents from English sole captured in these areas. Some of the highest prevalences of liver neoplasms found in Puget Sound were found in English sole from these areas.

Hepatic neoplasms, such as those described in fish, are inducible in mammals by a wide variety of organic chemicals (31). In fish, hepatocellular and cholangiocellular neoplasms similar to those seen in our study have been produced by exposure to methylazoxymethanol acetate (32), 4-dimethylamino-azobenzene, *N*-1-fluorenylacetamide, *o*-aminoazotoluene, diethylnitrosamine, dimethylnitrosamine (33), *N*-methyl-*N'*-nitrosoguanidine (34), and 7,12-dimethylbenz[a]anthracene (35). In fact, that fish may be even more predisposed to certain carcinogenic agents than are mammals is suggested by recent findings that DNA excision-repair with skin fibroblasts is distinctly low in fish in comparison to mammals (36). Thus, a wide variety of chemicals, including AHs, may be implicated in the etiology of hepatic neoplasms in the bottom-dwelling fish. It is also interesting to note that BaP is not a liver carcinogen in mammals, although lesions of the skin and other parts of the body have been produced in rodents (37). The question of whether BaP can produce liver neoplasms in English sole has not yet been answered; however, it was shown that liver carcinomas are produced in rainbow trout (*Salmo gairdneri*) after exposure to this carcinogen in the diet and via intraperitoneal injection (38).

Although little definitive information exists about the routes of exposure of bottom-dwelling fish to toxic chemicals, the Eagle Harbor and Mukilteo studies demonstrated that known mutagens and carcinogens were present in the invertebrates comprising the greater part of the stomach contents of English sole. Thus, a dietary route appears to be of some significance. A similar finding was made with white croaker (*Genyonemus lineatus*) from San Pedro Bay near Los Angeles, CA (Malins et al., manuscript submitted). The concentrations of chemicals in the food organisms in the stomachs of this species were generally higher than the sediment concentrations from where the fish were captured. For example, food organisms had concentrations of summed

AHs, PCBs, and DDT-related compounds which were 34, 8, and 58 times those in the sediment, respectively.

The problems associated with liver neoplasia in the Puget Sound bottom-dwelling fish do not occur in isolation; high prevalences of neoplastic lesions also have been found in other areas of the world having chemically contaminated sediments. Such lesions have been identified, for example, in Atlantic hagfish (*Myxine glutinosa*) from a fjord in Sweden (39), in Atlantic tomcod (*Microgadus tomcod*) from the Hudson River estuary (40), and in winter flounder (*Pseudopleuronectes americanus*) from Boston Harbor (41). Liver neoplasms have also been found in high prevalences in the freshwater bottom-dwelling fish, the brown bullhead (*Ictalurus nebulosus*), living in polluted rivers associated with the Great Lakes (42-44). It is clear, however, that further insight into the etiology of the neoplasms will require continued intensive study in the laboratory.

This project has been funded in part with Federal funds from the Department of Health and Human Services under Interagency Agreement number Y01-CP-40507, and by the Ocean Assessments Division, National Ocean Services, NOAA. The contents of this publication do not necessarily reflect the views or policies of the Department of Health and Human Services or of NOAA. We thank W. D. Gronlund, L. D. Rhodes, L. L. Johnson, O. P. Olson, D. R. Bunnell, A. J. Friedman, L. K. Moore, D. G. Burrows, R. G. Bogar, J. A. Werner, J. C. Drury, K. L. Grams, O. Maynes, D. L. Del Beccaro, C. A. Wigren, and R. W. Pearce for sample collection, processing, and analysis; P. A. Robisch, Dr. C. A. Krone, and P. Emry for preparation and fractionation of sediment extracts; and W. D. Weber, F. J. Jacques, M. L. Baumgaertner, J. C. Cesarone, and P. D. Plesha for injection and maintenance of experimental fish. Appreciation is also given to G. A. Blackledge and L. A. Wirick for typing; to J. Peacock and B. S. Kuhne for figures; and to P. A. Robisch, Dr. C. A. Krone, and Dr. E. Casillas for manuscript review.

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